Studies on the Substrate Specificity of the Proteinase of Equine Infectious Anemia Virus Using Oligopeptide Substrates†

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ABSTRACT: The proteinase of the equine infectious anemia virus (EIAV), a lentivirus closely related to human immunodeficiency virus (HIV), was purified from concentrated virus. The specificity of the enzyme was characterized using oligopeptides representing naturally occurring cleavage sites in the Gag and Gag–Pol polyproteins. The length of the substrate binding pocket was found to be 1–2 residues longer than that of HIV proteinases. Although the EIAV and HIV proteinases cleaved most of the peptides at the same bond, some were hydrolyzed by only the EIAV enzyme. Oligopeptides representing cleavage sites in the nucleocapsid protein were also found to be substrates of the EIAV proteinase. However, these peptides were not hydrolyzed by the HIV proteinases. While peptides representing the corresponding sequences in the first cysteine arrays of the nucleocapsid proteins of HIV-1 and HIV-2 were substrates of the proteinases, peptides representing the homologous sequences in the second Cys arrays were resistant against the proteolytic attack. A three-dimensional model of the EIAV proteinase built on the basis of homology with HIV-1 proteinase was used to interpret the differences. In addition to the oligopeptides representing cleavage sites in the Gag and Gag–Pol polyproteins, the EIAV proteinase was also able to cleave an oligopeptide mimicking a cleavage site in the transmembrane protein. Our results suggest that the specificity of lentiviral proteinases share common characteristics, although substantial differences may exist in hydrolysis of some peptides.

Retroviral proteinase (PR)[1] plays a crucial role in the virus life-cycle by processing the Gag and Gag–Pol polyproteins of immature virions [for review see Kräusslich and Wimmer (1988), Skalka (1989), Hellen et al. (1989), Oroszlan and Luftig (1990), Oroszlan and Tözsér (1990), and Kay and Dunn (1990)]. The proteinases of human immunodeficiency virus type-1 (HIV-1) and type-2 (HIV-2) have been intensively studied, as potential targets for chemotherapy. However, the proteinase of other lentiviruses has not been as extensively characterized.

Equine infectious anemia virus (EIAV) has been classified as a lentivirus (Gonda et al., 1978). It is closely related to HIV, on the basis of similar morphology [for review, see Gonda et al. (1988)], sequence, and genomic organization (Chiu et al., 1985, Rushlow et al., 1986; Stephens et al., 1986). The primary structure of the proteinase region of EIAV also shows a strong homology to the HIV proteinases (Weber, 1989). EIAV is the only lentivirus, for which capsid preparation has been developed (Roberts & Oroszlan, 1989); therefore, it is an important model of HIV. During EIAV capsid incubation, further proteolytic cleavage of the nucleocapsid (NC) protein was observed, and the viral proteinase was suggested to be responsible for these cleavages (Roberts & Oroszlan, 1989). The cleavage sites were determined by N-terminal amino acid sequencing and C-terminal analysis of the cleavage products and were found to be between cysteines and aromatic amino acid residues in the two cysteine arrays. On the basis of these findings, a novel function of the retroviral proteinase has been suggested (Roberts & Oroszlan, 1990; Roberts et al., 1991). Preliminary experiments indicate that the in situ cleavage of the NC protein is not a unique characteristic of the EIAV capsid; it also occurs in other retroviral cores (Roberts et al., 1991; and unpublished data).

While the viral proteinase is responsible for the processing of the Gag and Gag–Pol polyproteins, the Env polyprotein is cleaved into the functional surface glycoprotein (SU) and transmembrane (TM) protein by a cellular proteinase (Hunter & Swanstrom, 1990). Cell culture studies have shown that the EIAV TM protein is further processed into a glycoprotein traversing the membrane and a C-terminal protein located at the cytoplasmic side (Rice et al., 1990). Although the cleavage site is an unusual one (-His*Leu-), it was suggested that the viral proteinase was involved (Rice et al., 1990). This is similar to the processing of the cytoplasmic domain of murine leukemia virus transmembrane protein by the viral proteinase during virus maturation (Schultz & Rein, 1985).

Since the EIAV system is used as a model for HIV, it is important to characterize the specificity of EIAV PR and

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[1] Abbreviations: AMV, avian myeloblastosis virus; BLV, bovine leukemia virus; EIAV, equine infectious anemia virus; HIV-1 and HIV-2, human immunodeficiency virus type-1 and type-2; MMTV, mouse mammary tumor virus; RSV, Rous sarcoma virus; DTT, dithiothreitol; EDTA, ethylenediamine tetraaceticacid; RP-HPLC, reversed-phase high-performance liquid chromatography; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; MA, matrix protein; CA, capsid protein; NC, nucleocapsid protein; TF, transframe protein; PR, proteinase; RT, reverse transcriptase; IN, integrase; SU, surface glycoprotein; TM, transmembrane protein. Nomenclature of viral proteins is according to Leis et al. (1988).

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determine its similarity to and difference from that of HIV proteinases. Therefore, we purified the EIAV PR from virus, determined the required length of a peptide to be a good substrate of the enzyme, and compared the specificity of EIAV PR to that of HIV proteinases using oligopeptides representing the naturally occurring EIAV cleavage sites (Henderson et al., 1987). It was also our aim to investigate the in vitro processing of various oligopeptides representing determined cleavage sites in the nucleocapsid protein of EIAV and predicted sites for HIV-1 and HIV-2 (Roberts et al., 1991). A peptide representing the observed cleavage site in the transmembrane protein of EIAV (Rice et al., 1990) and several peptides from the predicted cleavage site region at the junction of reverse transcriptase (RT) and RNase H domains of both EIAV and HIV were also studied. The substrate kinetic measurements were analyzed with respect to the molecular model of EIAV PR. The details of this model are described in the following paper (Weber et al., 1993).

MATERIALS AND METHODS

Retroviral Proteinases. EIAV PR was purified in the following way: EIAV (Wyoming strain) was grown in chronically infected canine thymus (Cf2Th) cells and purified by sucrose density gradient centrifugation as previously described (Charman et al., 1976). Double banded concentrated virus was supplied by the Biological Product Laboratory, NCI–Frederick Cancer Research and Development Center, Frederick, MD. An aliquot of the concentrated (5000x) suspension of purified virus (1.2 mg/mL) in 10 mM Tris-HCl, pH 7.2, containing 1 mM EDTA and 100 mM NaCl, was disrupted by the addition of an equal amount of 20 mM Pipes, pH 7.0, containing 1 mM EDTA, 100 mM NaCl, 10% glycerol, 5% ethylene glycol, 0.5% Nonidet P-40, and 10 mM dithiothreitol (DTT). Proteinase activity was measured from this solution. The EIAV PR was purified from the virus by reversed-phase high-performance liquid chromatography (RP-HPLC). In a typical run, an equal amount of 8 M guanidine hydrochloride was added to 1 mL of 5000x concentrated virus, and the solution was acidified to pH 2 by the addition of trifluoroacetic acid (TFA). This solution was applied to a Vydac Protein C_4 column, and proteins were separated by RP-HPLC as previously described (Henderson et al., 1987). After lyophilization of the HPLC fractions, the proteinase was refolded in 200 μL of 20 mM Pipes–HCl buffer, pH 7.0, containing 10% glycerol, 5 mM DTT, 100 mM NaCl, and 0.5% NP-40. The fraction having the highest activity was used in the proteinase assays. Protein concentration was determined according to Bradford (1976). SDS–polyacrylamide gel electrophoresis was performed according to Laemmli (1970) and immunoblotting according to Towbin et al. (1979).

Purified recombinant HIV-1 PR expressed in *Escherichia coli* was available from previous studies (Louis et al., 1989; Wondrak et al., 1991). HIV-2 PR was chemically synthesized using solid-phase peptide synthesis (Copeland & Oroszlan, 1988) and then purified and refolded as described previously (Tözsér et al., 1992).

Active-site titration of the enzymes was performed using a phosphinic acid-type substrate based inhibitor [compound 3 in Grobelny et al. (1990)]. The K_i values were 0.4 nM for both HIV-1 PR and HIV-2 PR and 0.2 nM for EIAV PR.

Oligopeptide Substrates. Oligopeptides were synthesized by solid-phase peptide synthesis on a Model 430A automated peptide synthesizer (Applied Biosystems, Inc.) or a semiautomatic Vega peptide synthesizer (Vega-Fox Biochemicals) using *tert*-butoxycarbonyl chemistry and purified by RP-HPLC (Copeland & Oroszlan, 1988). Amino acid compositions of the peptides were determined by amino acid analysis on either a Durrum D-500 or a Waters Pico-Tag analyzer. Stock solutions and dilutions were made in distilled water or in 10 mM DTT, and the proper peptide concentration was determined by amino acid analysis.

Enzyme Assay. The assays were performed in 0.25 M potassium phosphate buffer, pH 5.6, containing 7.5% glycerol, 5 mM DTT, 1 mM EDTA, 0.2% Nonidet P-40, and 2 M NaCl as previously described (Tözsér et al., 1991a,b, 1992). The reaction mixture was incubated at 37 °C for various time intervals and was stopped by the addition of guanidine hydrochloride (6 M final concentration). The solution was acidified to pH 2 by the addition of TFA and injected onto a Nova-Pak C_{18} reversed-phase chromatography column (3.9 × 150 mm) using a WISP 710A automatic injector (both from Waters Associates, Inc.). Substrates and the cleavage products were separated using acetonitrile gradient (0–100%) in water, in the presence of 0.05% TFA. The cleavage of peptides was monitored at 206 nm, and the peak areas were integrated using Waters 745B data module. Amino acid analysis of collected peaks was used to confirm the cleavage sites in the substrates. When required, sequence analysis was carried out to verify the cleavage site. The substrate concentrations used for the kinetic measurements were in the range of 0.02–3 mM, depending on the approximate K_m values. Kinetic parameters were determined at less than 20% substrate turnover (using 1–4-h incubation time depending on the degree of hydrolysis) by fitting the data to the Michaelis–Menten equation using the Gauss–Newton method. The computer program (Enz 5.0) was written and kindly provided by M. Fivash and J. Racheff of the Data Management Services, Inc., NCI–FCRDC, Frederick, MD. The asymptotic standard errors were below 20%. Substrate hydrolysis followed Michaelis–Menten kinetics in the concentration range of substrates used.

Molecular Modeling. The molecular model of EIAV PR was built as described in the following paper (Weber et al., 1993). The computer graphics program FRODO (Jones, 1978) was run on an Evans and Sutherland computer graphics system PS 390 or on an ESV10 to display the model structure for interpretation of peptide cleavage measurements.

RESULTS AND DISCUSSION

Purification of the EIAV Proteinase. EIAV PR was purified by RP-HPLC (Figure 1) and refolded as described in Materials and Methods. In a typical experiment, the recovered activity was 37%. The proteinase eluted at 44% acetonitrile, in front of p26. The increase in specific activity compared to the disrupted virus was 24-fold. SDS–polyacrylamide gel electrophoresis showed a single band migrating as an ~12-kDa protein (Figure 2) in agreement with the theoretical molecular weight based on 104 amino acid residues (Roberts et al., 1993). In a separate immunoblotting experiment the protease fraction reacted with anti-EIAV PR antibody (kindly provided by Dr. N. Rice), produced against a synthetic peptide representing the C terminus of the proteinase (data not shown).

Dependence of the Substrate Hydrolysis on the Substrate Length. The nonapeptide substrate, H-Val-Ser-Gln-Asn-Tyr*Pro-Ile-Val-Gln-NH_2 mimicking the cleavage site between the MA and CA proteins of HIV-1 (SP-211, asterisk indicates the site of cleavage) was found to be a good substrate not only for HIV-1 and HIV-2 proteinases (Copeland &

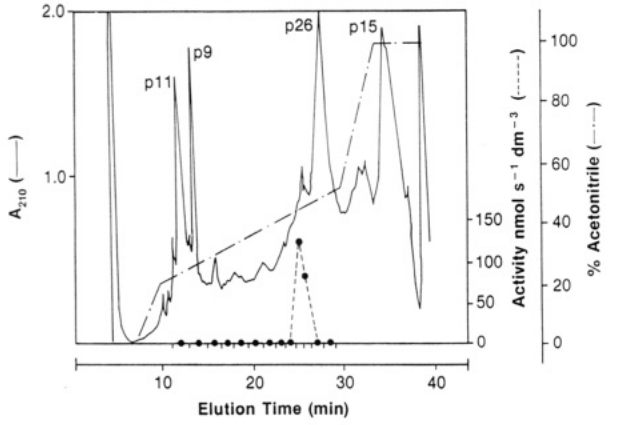

FIGURE 1: Purification of EIAV proteinase from virus by RP-HPLC. Proteolytic activity of the fractions after refolding was assayed using an oligopeptide representing the MA/CA cleavage site in HIV-1 (SP-211). Gag proteins p11 (NC), p9, p26 (CA), and p15 (MA) have been identified by SDS–polyacrylamide gel electrophoresis.

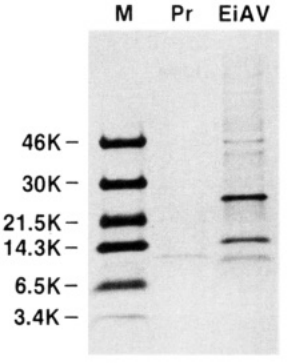

FIGURE 2: SDS–polyacrylamide gel electrophoresis of purified EIAV proteinase. Lanes as indicated. Molecular size markers (M): 46K, ovalbumin; 30K, carbonic anhydrase; 21.5, trypsin inhibitor; 14.3K, lysozyme; 6.5, aprotinin; 3.5K, insulin (b) chain.

Table I: Dependence of the Substrate Hydrolysis by EIAV PR on the Substrate Length

	peptide	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})	IC_{50}[a] (mM)
1.	VSQNY*PIVQ[b]	0.31	3.7	11.9	
2.	SQNY*PIVQ	2.2	1.7	0.8	
3.	QNY*PIVQ[c]		<0.01	ND[d]	~1.5[e]
4.	VSQNY*PIV	0.04	0.4	10.0	
5.	SQNY*PIV[c]		<0.01	ND	0.27

[a] IC_{50} values were measured as inhibition of the cleavage of SP-211. [b] Substrate previously designated as SP-211 representing the cleavage site between the matrix and capsid proteins in HIV-1 (Copeland & Oroszlan, 1988). The actual or expected cleavage sites are indicated by asterisks. [c] The degree of hydrolysis of these peptides was very small during a 24-h incubation. Other shorter peptides which were tested from the complete series (Tözsér et al., 1991b), but were not hydrolyzed by EIAV PR during a 24-h incubation: QNY*PIV, VSQNY*PI, SQNY*PI, QNY*PI, VSQNY*P. [d] Not determined. [e] This peptide gave ~50% inhibition at 1.5 mM, the highest concentration used.

Oroszlan, 1988) but also for EIAV PR (Copeland et al., 1990). A series of sequentially shortened analogs of SP-211 was used to study the effect of substrate length on peptide hydrolysis by EIAV PR (Table I). The same series was previously used to study the substrate binding sites of HIV-1 PR and HIV-2 PR (Tözsér et al., 1991b). The removal of the P5 Val [notation according to Schechter and Berger (1967)] resulted in a dramatic increase in K_m value and the decrease of k_{cat}. A similar increase in K_m was observed with HIV proteinases, but due to the concomitant increase of k_{cat} the specificity constants (k_{cat}/K_m) did not decrease dramatically (Tözsér et al., 1991b). The removal of P4′ Gln (peptide 4) resulted in a substantial decrease in both the K_m and k_{cat} values in such a way that the specificity constant remained similar to the original value obtained with the nonapeptide. The further stepwise removal of the P4 Ser and P4′ Gln of peptide 2 (Table I) resulted in peptide 3 and 5, respectively, which were very inefficient substrates for EIAV PR (Table I). The same peptides were previously found to be hydrolyzed by both HIV-1 and HIV-2 proteinases at a reasonable rate (Tözsér et al., 1991b). Truncated peptides 3 and 5 inhibited the cleavage of the nonapeptide SP-211 by EIAV PR. The IC_{50} values for those peptides shown in Table I suggest that the lack of hydrolysis is due to nonproductive binding, as found for HIV proteinases with shorter peptides (Tözsér et al., 1991b). Together, the results suggest that the substrate binding site of EIAV PR may be somewhat more extended than the binding site of HIV proteinases.

On the basis of molecular modeling, the overall structure of EIAV PR is predicted to be similar to that of HIV proteinases. The most significant exception is the presence of additional residues in the region leading to the tip of the flap and the turn between HIV-1 β strands a′ and b′ (Weber et al., 1993). In this region the EIAV PR more closely resembles the Rous sarcoma virus (RSV) PR in which the region leading to the flap is also extended by 5 residues as compared to HIV proteinases. The molecular model of RSV PR (Grinde et al., 1992) and EIAV PR (Weber et al., 1993) suggested that these additional residues form part of the subsites S4 and S4′ and provide interactions with longer substrates. This would explain the preference of EIAV PR for a longer substrate, extending P5 to P4′ (Table I) compared to the six-to-seven-residue minimal length (P4 to P3′) for substrates of HIV proteinases (Darke et al., 1988; Moore et al., 1988; Tözsér et al., 1991b). It should be pointed out that avian myeblastosis virus (AMV) PR also appears to have a longer substrate recognition site: removal of the P5 residue from a substrate reduced the relative activity about half, and the additional stepwise removal of P4 and P3′ residues resulted in nonhydrolyzable peptides (Strop et al., 1991).

Hydrolysis of Oligopeptides Representing Naturally Occurring Cleavage Sites at the Junctions of Functional Domains of the Gag and Gag–Pol Polyproteins by EIAV Proteinase. Equal length oligopeptides (all decapeptides) representing determined cleavage sites in the Gag and Gag–Pol polyproteins of EIAV (see Figure 3) (Henderson et al., 1987; Roberts et al., 1993) were studied as substrates of EIAV PR and also for comparison as substrates of HIV-2 PR (Table II). Except for the peptide representing the $p15^{pol}$/IN cleavage site, all were substrates of the EIAV PR. Besides the oligopeptides representing cleavage sites in its own Gag–Pol polyprotein, EIAV PR can cleave all nine representative peptides mimicking HIV-1 Gag–Pol cleavage sites, and previously found to be substrates of both HIV-1 PR and HIV-2 PR (Tözsér et al., 1991a), at the same sites as the HIV enzymes (data not shown). On the other hand, three peptides representing EIAV cleavage sites were not substrates for HIV proteinases (Table II). The most dramatic difference is with peptide 4 (NC/p9 cleavage site, see also Figure 3), which is an excellent substrate of EIAV PR but is not hydrolyzed by HIV-2 PR. This is probably due to the far higher preference for Thr at P2 position by EIAV PR as compared to HIV proteinases (Weber et al., 1993; Tözsér et al., 1992) and a preference for Lys at P4. The residues at positions P3 to P3′ of peptide 4 are among those that form the best substrates for EIAV PR in the tested series of single amino acid substitutions of SP-211 (Weber et al.,

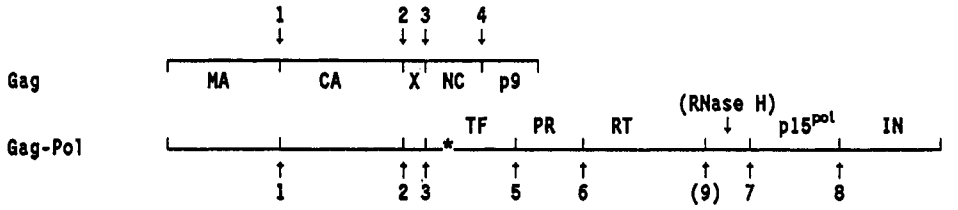

FIGURE 3: Cleavage sites in EIAV Gag–Pol polyproteins. The cleavage sites are taken from Henderson et al. (1987) and Roberts et al. (1993). The putative cleavage site between the RT and N-terminal side of the RNase-H domain (site 9) has not been determined by sequencing of the viral proteins.

Table II: Kinetic Parameters Obtained for EIAV and HIV-2 Proteinases Using Oligopeptide Substrates Representing Cleavage Sites in Gag and Gag–Pol Polyproteins of EIAV[a]

		EIAV PR		HIV-2 PR	
place of cleavage	sequence of peptide	K_m (mM)	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})
1. MA/CA	PSEEY*PIMID	0.08	6.1	0.8	3.0
2. CA/X	QKMML*LAKAL	<0.03	1.3	<0.3	0.4
3. X/NC	LAKAL*QTGLA	ND[b]	<0.02	not hydrolyzed[c]	
4. NC/p9	QKQTF*PIQQK	0.27	10.4	not hydrolyzed[c]	
5. TF/PR	GQFVG*VTYNL	ND	<0.01	not hydrolyzed[c]	
6. PR/RT	AKLVL*AQLSK	0.02	0.4	0.02	0.7
7. RT/p15pol	KEEIM*LAYQG	<0.03	2.5	<0.03	0.5
8. p15pol/IN	STGVF*WVENI	not hydrolyzed[c]		not hydrolyzed[c]	

[a] Cleavage sites are taken from Henderson et al. (1987) (sites 1, 2, 3, and 4) and Roberts et al. (1993) (sites 6, 6, 7, and 8). The cleavage sites found by sequencing of the viral protein products are marked with asterisk. If the peptide representing the naturally occurring cleavage site was hydrolyzed, the cleavage site in the peptide was found to be the expected one on the basis of amino acid analysis of the cleavage products. [b] ND, not determined. [c] These peptides were not hydrolyzed during a 24-h incubation with the enzyme.

1993). Analysis of the molecular model of EIAV PR and the activity toward peptides with single amino acid substitutions in the SP-211 peptide have shown that EIAV PR and HIV-1 PR differ most in the preferences for the P4 and P2 positions. Peptides 1 (MA/CA) and 7 (RT/p15pol) are substantially better substrates for EIAV PR than for HIV-2 PR, and both contain Glu at positions P2 and P3 or P3 and P4 (Table II). These differences may be explained by the presence of the neutral residue Thr 30 in subsites S4 and S2 of EIAV PR, instead of the negatively charged Asp 30 in HIV PR which would be unfavorably close to Glu at P4 or P2. The results obtained with the peptides representing EIAV and HIV-1 Gag–Pol cleavage sites suggest that the substrate specificity of EIAV PR is broader than that of HIV PRs.

In general, oligopeptides representing naturally occurring cleavage sites at the junctions of functional protein domains of HIV-1, HIV-2, bovine leukemia virus (BLV), AMV, and mouse mammary tumor virus (MMTV) polyproteins were found to be substrates of the respective proteinases (Darke et al., 1988; Tözsér et al., 1991a; Andreánsky et al., 1991; Strop et al., 1991; Menendez-Arias et al., 1992). Using EIAV PR with peptides mimicking EIAV Gag–Pol cleavage sites (Table II), there was a great variation in the observed values of K_m (0.02–0.27 mM) and k_{cat} (0.01–10.1 s^{-1}). Similar results were obtained for the HIV-1 cleavage sites/HIV-1 PR and HIV-2 cleavage sites/HIV-2 PR systems (Tözsér et al., 1991a).

The cleavage sites of the immunodeficiency viruses as well as other retroviral cleavage sites have been classified on the basis of amino acid sequence around the scissile bond (Henderson et al., 1988; Pettit et al., 1991). Since the cleavage site at the amino terminus of the capsid (CA) protein is always a type 1 site having Pro at the P1′ position, a specific function has been suggested (Oroszlan et al., 1978; Oroszlan & Gilden, 1979; Pettit et al., 1991). It is of interest to note that in homologous systems of EIAV (Table II) HIV-1 and HIV-2 (Tözsér et al., 1991a), BLV (Andreansky et al., 1991; Blaha et al., 1992), and MMTV (Menendes-Arias et al., 1992) the peptides representing these cleavage sites are usually among the best substrates. However, neither the location in the polyprotein nor the type of cleavage site shows obvious correlation with the kinetics of the respective peptide hydrolysis.

Table III: Proteolytic Processing of Oligopeptides Representing Determined or Predicted Cleavage Sites in the Nucleocapsid Protein of EIAV, HIV-1, and HIV-2

		EIAV-PR	
location of cleavage site	sequence of peptide	K_m (mM)	k_{cat} (s^{-1})
EIAV NC-1[a]	AAQTC*YNCGK	0.35	0.2[b]
EIAV NC-2[c]	APKVC*FKCKQ	0.6	0.2[b]
HIV-1 NC-1[a]	KMVKCF*NCGK	0.47	1.2[d]
HIV-2 NC-1[a]	KPIKCW*NCGK	0.02	0.02[e]

[a] Cleavage site in the first Cys array (Roberts et al., 1991). [b] These peptides were not hydrolyzed by the HIV-1 PR or HIV-2 PR. [c] Cleavage site in the second Cys array (Roberts et al., 1991). [d] This peptide was also hydrolyzed by HIV-1 PR, with a k_{cat} value of 0.01. [e] This peptide was also hydrolyzed by HIV-2 PR, with a k_{cat} value of 0.01.

Hydrolysis of Oligopeptides Having the Sequence of Nucleocapsid Protein Cleavage Sites. Oligopeptides (EIAV NC-1 and NC-2) having the sequence of the cleavage site in the first and second cysteine arrays of the nucleocapsid protein of EIAV (Roberts et al., 1991) were studied as substrates for EIAV PR and HIV proteinases (Table III). These peptides were hydrolyzed at the expected -Cys*Tyr- and -Cys*Phe- bonds. SP-346, a specific retroviral PR inhibitor (Copeland et al., 1990), inhibited these cleavages. The kinetic parameters for the hydrolysis of these peptides are listed in Table III. A longer peptide representing the C-terminal part of the NC protein including the complete second Cys array was also synthesized (APKVC*FKCKQPGHFSKQCRSVPKNGKQGAQGRPQKQTF) and was found to be hydrolyzed by the EIAV PR at the -Cys*Phe- bond (data not shown). On the other hand, none of the EIAV "NC peptides" were cleaved by either HIV-1 PR or HIV-2 PR (k_{cat} values are estimated to be less than 0.01 s^{-1}). This is predicted to be at least partly due to the large difference for the preference at the P4 and P2 positions: both Thr and Val are far better accommodated in the S2 pocket of EIAV PR than in that of HIV proteinases,

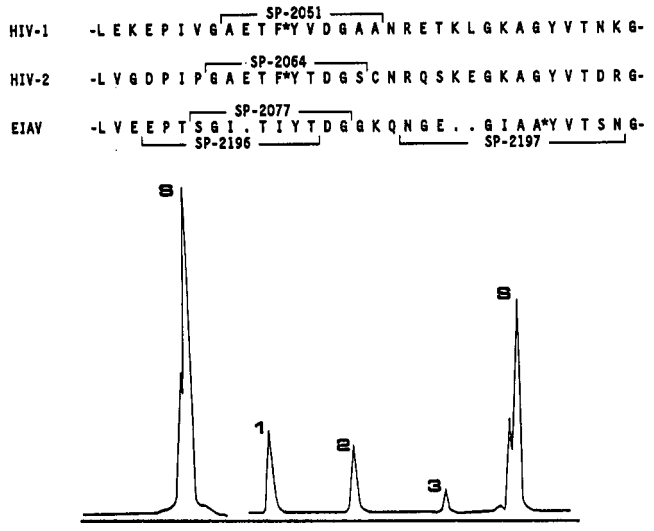

FIGURE 4: (A, top) Sequence of the segment of reverse transcriptase of HIV-1_{BH10}, HIV-2_{NIHZ}, and $EIAV_{CG}$ containing the determined and predicted cleavage sites to produce RNase H. SP-2051, SP-2064: oligopeptides representing the cleavage site in HIV-1 and HIV-2 reverse transcriptase, found to be hydrolyzed by both HIV-1 and HIV-2 proteinases (Tozser et al., 1991a). (B, bottom) Among the peptides representing the homologous region and its surroundings in EIAV, only SP-2197 was hydrolyzed by EIAV and HIV PRs. The RP-HPLC profile of the reaction mixture containing 0.4 mM substrate is shown after 18 h of incubation at 37 °C in the absence (at left) and presence (at right) of 6 nM EIAV PR. Peaks were identified by amino acid analysis as follows: 1, YVTS; 2, NGEGIAA; 3, NGEGIAAYVTS; S, NGEGIAAYVTS.

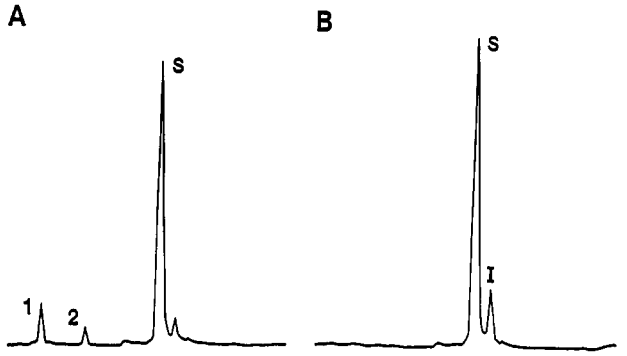

FIGURE 5: Cleavage of the oligopeptide representing the transmembrane protein cleavage site of EIAV by EIAV proteinase. The RP-HPLC profile of the reaction mixture containing 0.2 mM substrate is shown after 12 h of incubation at 37 °C in the presence of 5 nM purified EIAV PR and in the absence (A) and presence (B) of 20 μM proteinase inhibitor (SP-346). Peaks were identified by amino acid analysis as follows: 1, AQHNIH; 2, LAGVT; S, AQHNIHLAGVT. "I" marks the peak of inhibitor SP-346.

while hydrophobic residues such as Ala, Val, and Ile are preferred in S4 (Weber et al., 1993).

Oligopeptides representing analogous regions in the nucleocapsid proteins of HIVs (HIV-1 NC-1 and HIV-2 NC-1; Table III) were also found to be hydrolyzed by HIV proteinases and EIAV PR; however, cleavage occurred at the -Phe*Asn- and -Trp*Asn-bonds instead of the predicted (Roberts et al., 1991) -Cys*Phe- and -Cys*Trp-bonds. This "shift" may be partially due to the presence of Lys adjacent to Cys (see Table III). Substitution of Asn with Lys at the P2 position of substrate SP-211 prevented hydrolysis by HIV proteinases (Tözsér et al., 1992) and also by EIAV PR (Weber et al., 1993). On the other hand, while replacement of the P1 Tyr to Cys produced an inefficient substrate of both HIV PR and EIAV PR (Tözsér et al., 1992; Weber et al., 1993), at P2 position it fits very well in the pocket of EIAV and HIV proteinases and forms a better substrate for EIAV PR compared to HIV PR (Weber et al., 1993). However, it should be pointed out that intramolecular interactions between adjacent side chains of the substrates may also contribute to the specificity at a given subsite as described for HIV PR (Tözsér et al., 1992).

Prediction of the Cleavage Site between Reverse Transcriptase and RNase H Domains of EIAV. It has been found that there is an incomplete processing of the 66-kDa RT of HIV-1 resulting in the formation of a 52-kDa RT and the RNase H (Veronese et al,. 1986). Similar processing has been found in HIV-2 (DeVico et al., 1989) and in EIAV (DeVico et al., 1991; Roberts et al., 1993). The peptide representing the HIV-1 cleavage site has been found to be a substrate of HIV-1 PR and HIV-2 PR (Tözsér et al., 1991a) and can also be cleaved at the same site by EIAV PR (not shown). To determine the putative cleavage site in the EIAV RT, an oligopeptide was synthesized having the sequence of the region homologous to the respective HIV-1 sequence (SP-2077) (Figure 4, top). However, this peptide was not a substrate of EIAV PR. To study whether the surrounding sequences contain a cleavage site, two additional peptides (SP-2196, SP-2197) were synthesized (Figure 4, top) and assayed as substrates for EIAV PR. One of them, SP-2197 was hydrolyzed at the -Ala*Tyr- bond as a major site (Figure 4, bottom). However, the recovery of a small amount of another product (NGEGIAAY) suggested an additional minor cleavage at the -Tyr*Val-bond. Although HIV-2 PR cleaved the SP-2197 peptide only at the -Ala*Tyr-site, HIV-1 PR also produced the minor cleavage product (not shown).

Retroviruses mutate at a very rapid rate (Holland et al., 1982). Since the proteolytic processing of the Gag–Pol polyproteins is required for infectivity, not only should the proteinase region remain homologous but also mutations at the cleavage sites may result in noninfectious virions. Therefore, it is of interest to compare the exact location and sequence of the lentiviral cleavage sites. The strong homology between the HIV-1 and HIV-2 results in cleavage sites with homologous sequences at the junctions of major protein domains (Oroszlan & Tözsér, 1990). However, the position of the X/NC cleavage site (see Figure 3) seems to be shifted due to the substantial sequence divergence (Henderson et al., 1988). When we analyzed a putative cleavage site in the RT of EIAV, a similar shift was indicated: the putative EIAV cleavage site was found to be close and shifted downstream of the region homologous to the HIV-1 and HIV-2 RT cleavage sites. It is tempting to speculate that mutation of the P1 Phe to β-branched Ile prevented the substrate hydrolysis at the position equivalent to the cleavage sites of HIV-1 and HIV-2 since, as noted earlier, β-branched residues cannot occur at P1 positions of retroviral proteinases (Kotler et al., 1988; Phylip et al., 1990). We suggest that due to the selective evolutionary pressure the mutation of a glycine to alanine created another cleavage site nearby (see Figure 4, top).

Cleavage of an Oligopeptide Representing the Cleavage Site in EIAV Transmembrane Protein. An oligopeptide representing the cleavage site in the TM protein of EIAV (Rice et al., 1990) has been synthesized (SP-1072, AQHNIH*LAGVT). This peptide was hydrolyzed by the RP-HPLC-purified EIAV PR at the -His*Leu-bond (Figure 5). This cleavage was inhibited by SP-346, a specific inhibitor of HIV and EIAV proteinases (Copeland et al., 1990), indicating that the retroviral enzyme is responsible (Figure 5). In addition to the processing of the Gag–Pol polyproteins, recently other functions of the retroviral proteinase have been suggested, which include the cleavage of cellular proteins like vimentin

(Shoeman et al., 1990) and transcriptional factor NF-κB (Riviere et al., 1991). Studies of the processing of the transmembrane protein in EIAV suggested an additional role of PR in truncating the cytoplasmic domain, although the exact function of this truncation is not known (Rice et al., 1990). The hydrolysis of the oligopeptide representing this cleavage site by purified EIAV PR and the inhibition of this cleavage by a specific inhibitor support the involvement of EIAV PR in this cleavage. It would be very interesting to see whether similar processing could occur in HIV transmembrane proteins, although the EIAV cleavage site peptide was not hydrolyzed by HIV proteinases.

CONCLUSIONS

Our results suggest that lentiviral proteinases share common characteristics in their specificities. HIV-1 PR and HIV-2 PR have shown similar specificities on various oligopeptides in our assay system (Tözsér et al., 1991a,b, 1992), although others found more dramatic differences using particular substrates or inhibitors (Tomasselli et al., 1990). As the divergence is increased between two proteinases, one would expect more substantial differences in kinetics of substrate hydrolysis, or even shifts in the site of cleavage. The proteinase of EIAV, a lentivirus, is still a close relative of the HIV proteinases and only 5 out of 20 residues differ in the predicted substrate binding region (Weber et al., 1993). In fact, these enzymes share many common characteristics in specificity. Although some of the peptides studied were hydrolyzed only by the EIAV PR (suggesting its broader specificity), if a peptide was hydrolyzed by both enzymes, the cleavage took place at the same bond. The molecular explanation of this phenomenon lies in the nature of the enzyme substrate interaction. Since about seven consecutive substrate residues are recognized by the enzyme, changes in one or in a few binding subsites of the enzyme due to mutations may alter but do not necessarily abolish the enzyme activity. This should be very advantageous for an enzyme encoded in rapidly mutating RNA.

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